Peptide α-Keto Ester, α-Keto Amide, and α-Keto Acid Inhibitors of Calpains and Other Cysteine Proteases

Zhaozhao Li,[†] Girish S. Patil,[†] Zbigniew E. Golubski,[†] Hitoshi Hori,[†,§] Kamin Tehrani,[†] J. E. Foreman,[‡] David D. Eveleth,[‡,∥] Raymond T. Bartus,[‡,⊥] and James C. Powers*,[†]

School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, Georgia 30332-0400, Cortex Pharmaceuticals, Inc., 15241 Barranca Parkway, Irvine, California 92718, and Department of Biological Chemistry, University of California, Irvine, California 92717

Received March 10, 1993[⊕]

A series of dipeptidyl and tripeptidyl α-keto esters, α-keto amides, and α-keto acids having leucine in the P_2 position were synthesized and evaluated as inhibitors for the cysteine proteases calpain I, calpain II, cathepsin B, and papain. In general, peptidyl α-keto acids were more inhibitory toward calpain I and II than α-keto amides, which in turn were more effective than α-keto esters. In the series Z-Leu-AA-COOEt, the inhibitory potency decreased in the order: Met (lowest K_I) > Nva > Phe > 4-Cl-Phe > Abu > Nle (highest K_I) with calpain I, while almost the reverse order was observed for calpain II. Extending the dipeptide α-keto ester to a tripeptide α-keto ester yielded significant enhancement in the inhibitory potency toward cathepsin B, but smaller changes toward the calpains. Changing the ester group in the α-keto esters did not substantially decrease K_I values for calpain I and calpain II. N-Monosubstituted α-keto amides were better inhibitors than the corresponding α-keto esters. α-Keto amides with hydrophobic alkyl groups or alkyl groups with an attached phenyl group had the lower K_I values. N,N-Disubstituted α-keto amides were much less potent inhibitors than the corresponding N-monosubstituted peptide α-keto amides. The peptide α-keto acid Z-Leu-Phe-COOH was the best inhibitor for calpain I (K_I = 0.0085 μM) and calpain II (K_I = 0.0057 μM) discovered in this study. It is likely that the inhibitors are transition-state analogs and form tetrahedral adducts with the active site cysteine of cysteine proteases and form hydrogen bonds with the active site histidine and possibly another hydrogen bond donor in the case of monosubstituted amides. Several inhibitors prevented spectrin degradation in a platelet membrane permeability assay and may be useful for the treatment of diseases which involve neurodegeneration.

Introduction

Calpains are calcium-dependent cysteine proteases which are widely distributed in mammalian cells, with platelets being a particularly rich source of the enzyme. There are two distinct classes of calpains: the first class requires micromolar concentrations of calcium for optimal enzymatic activity and is referred to as calpain I or μ-calpain. A second class requires millimolar concentrations of calcium and is referred to as calpain II or m-calpain.[1-3] Calpains have many possible biological roles including the development of long-term memory, the breakdown of neurofilaments at axon terminals, muscle protein turnover, breakdown of membrane proteins, cytoskeletal modification and cleavage of surface proteins during platelet activation, the metabolism of neuropeptides, and the regulation of meiosis.[4] Since calpains are involved in such a diversity of important physiological processes, calpain inhibitors may be useful for the treatment of a variety of disease states especially those involving neurodegeneration such as stroke.[5]

A wide variety of inhibitor structures have now been reported to effectively inhibit calpains. These include transition-state inhibitors, a variety of irreversible inhibitors, calmodulin antagonists, and polyamines.[6-8] Transition-state inhibitors are generally peptide derivatives containing an electrophilic carbonyl group in place of the scissile peptide bond of the substrate. Examples of transition-state inhibitors for cysteine proteases include peptide aldehydes, peptide fluoroalkyl ketones, and dicarbonyl derivatives.[9] Most of these transition-state inhibitors contain an electronegative functional group adjacent to the carbonyl group which corresponds to the scissile peptide carbonyl group and generally are inhibitors for both serine and cysteine proteases. The peptide aldehydes leupeptin and antipain from *actinomycetes* were some of the first compounds discovered to inhibit calpain moderately.[10] Subsequently, peptide aldehyde sequences based on the substrate specificity of calpain toward AMC substrates were synthesized and found to be potent reversible inhibitors of calpain I and calpain II with K_I values as low as 36 nM.[11] The most potent inhibitors are dipeptide aldehydes, and those with a P_1 Met were slightly more potent than the Nle and Phe derivatives. The mechanism of inhibition involves formation of a tetrahedral hemiketal (hemiacetal with aldehydes) or a hemithioketal enzyme–inhibitor complex upon reaction of the inhibitor carbonyl group with either the active site serine or cysteine residue, respectively.

We initially reported the peptidyl α-keto ester functional group as an inhibitor of serine proteases.[12] The design was based on the crystal structure of trypsin complexed with the keto acid (4-amidinophenyl)pyruvate (APPA).[13-15] In this structure, the active site Ser-195 of trypsin has added to the ketone carbonyl group of APPA to give a tetrahedral adduct with the oxyanion stabilized by in-

[†] Georgia Institute of Technology.
[‡] Cortex, Inc.
[§] Present address: Dept. Of Biol. Sci. & Technology, Univ. Of Tokushima, Minamijosanjima, Tokushima 770, Japan.
[∥] University of California, Irvine.
[⊥] Present address: Alkermes Inc., 64 Sidney St., Cambridge, MA 02139-4234.
[⊕] Abstract published in *Advance ACS Abstracts*, October 1, 1993.

0022-2623/93/1836-3472$04.00/0

teractions with trypsin's oxyanion hole. The benzamidine portion of the inhibitor is interacting with the S_1 pocket or primary substrate binding site of trypsin,[16] while the carboxylate of APPA is forming a salt link with the active site His-57. We then incorporated the α-keto acid and α-keto ester functional group into peptide inhibitors for chymotrypsin-like enzymes and elastases.[12] Additional peptide α-keto ester structures were subsequently investigated with other serine proteases and extended to cysteine proteases including calpain by a number of other groups.[15,17-20] In addition, the natural thrombin inhibitors cyclotheonamide A and B from a marine sponge contain the α-keto amide functional group.[21] Peptide α-keto ester inhibitors are frequently more potent inhibitors than the corresponding peptide trifluoromethyl ketones.

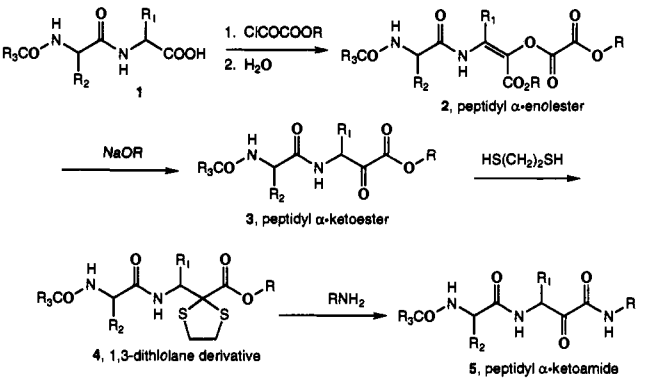
In recent years, peptide derivatives containing electrophilic carbonyl groups have been described as inhibitors for cysteine proteases, particularly papain, cathepsin B, and calpain. In comparing a variety of peptide inhibitors for papain and cathepsin B, dipeptidyl α-keto ester inhibitors were found to be more potent than the corresponding keto acids, which in turn were better than ketones.[22] A similar study with calpain showed that peptide aldehydes were more effective inhibitors than α-keto acids or α-keto esters, while peptide trifluoromethyl ketones were much less potent calpain inhibitors.[19]

In this paper we report the design and synthesis of a series of dipeptidyl and tripeptidyl α-keto ester, α-keto amide, and α-keto acid inhibitors for calpain I and calpain II. Only a few peptide derivatives containing electrophilic carbonyl groups have previously been reported as calpain inhibitors,[19] although other types of inhibitors such as ester and amide derivatives of E64c have been extensively studied with calpain.[23] The inhibitors which we report have leucine in the P_2 position, and varying P_1 and P_3 residues; N-terminal protecting groups; and varying ester groups and N-substituents on the α-keto amide inhibitors. Only one peptide α-keto amide derivative has previously been reported as a cysteine protease inhibitor,[22] although 3-amino-2-oxo-4-phenylbutanoic acid amides have recently been reported as a new class of aminopeptidase inhibitors.[24] We also report a new method for the synthesis of α-keto amides.

Chemistry

Amino acid methyl ester hydrochlorides were prepared on a scale of 0.1–1.0 mol in high yield by the method of Brenner.[25,26] The amino acid methyl esters were than acylated to form *N*-acyl amino acid methyl esters which were then hydrolyzed to *N*-acyl amino acids.[27] *N*-Acyl dipeptide and tripeptide methyl esters were synthesized stepwise via the HOBt-DCC method in a DMF solution.[28] *N*-Acyl dipeptides (1) and tripeptides were obtained by hydrolysis of the appropriate methyl esters. A modified two step Dakin–West reaction (Scheme I) was used to prepare peptidyl α-enol esters (2) in moderate yield.[29,30] The varying ester alkoxy groups in the α-enol esters were introduced by using the appropriate alkyl oxalyl chloride derivative.[31] Peptidyl α-keto esters (3) were then prepared by reacting the α-enol esters (2) with appropriate alkoxides in moderate to high yield and due to the mechanism of the Dakin–West reaction are all diastereomeric at the P_1 α-carbon atom. Direct reaction of the α-keto esters with alkylamines led to mixtures of products, and thus the peptidyl α-keto amides were prepared by protecting the

Scheme I. Preparation of Peptidyl α-Keto Esters and α-Keto Amides



α-carbonyl group of the α-keto ester first as a 1,3-dithiolane derivative (4). Reaction of the 1,3-dithiolane derivative with the appropriate amine yielded the α-keto amide (5) in moderate yield. Interestingly, the 1,3-dithiolane protection group was lost during the reaction or workup. Peptidyl α-keto acids were prepared by alkaline hydrolysis of the α-keto esters in high yield.

Results and Discussion

The substrate specificity of calpain has been studied with natural peptides, synthetic peptide substrates, and proteins.[32,33] With peptides, the site of cleavage indicates a preference for Leu or Val at P_2 and for Arg, Lys, Met, and Tyr at P_1. Phe, Trp, Leu, and Val predominate at P_3. Pro never appears in either P_1 or P_1'. A few synthetic peptide AMC substrates have also been tested with calpains; for example, Suc-Leu-Met-AMC is 16-fold better than Suc-Val-Met-AMC, indicating the importance of Leu at P_2. A similar trend is observed with Suc-Leu-Tyr-AMC and Suc-Val-Tyr-AMC. The P_2 residue seems to be of more significance than P_1. Thus, we chose Leu as the P_2 residue in all the dipeptide and tripeptide α-keto esters, α-keto amides, and α-keto acids which we synthesized.

Peptidyl α-Keto Esters. The inhibitory activities of α-keto esters toward calpain I, calpain II, cathepsin B, and papain are summarized in Table I. In the Z-Leu-AA-COOEt series (compounds 8, 12, 23–26), we found that the inhibitory potency increased (K_I value decreased) in the order: Nle (7.0 μM) < Abu (4.5) < 4-Cl-Phe (4.0) < Phe (1.8) < Nva (1.4) < Met (1.0) with calpain I, while almost the reverse order was observed with calpain II: Met (1.5 μM) < Nva (1.2) < Phe and Abu and 4-Cl-Phe (0.4) < Nle (0.18). All of the dipeptide α-keto esters in this series were more effective inhibitors for calpain than simple N-protected amino acid α-keto esters such as Z-Phe-CO_2-Et and the trifluoromethyl ketone Bz-Phe-CF_3 reported earlier.[19] Clearly, the inhibitory potency toward calpains increases when the inhibitors contain a P_2 residue. The dipeptide calpain inhibitor Z-Val-Phe-CO_2Et has a K_I value of 0.4 μM[19] with calpain, which is identical to the value of 0.4 μM which we measured for the related α-keto ester Z-Leu-Phe-CO_2Et with calpain II.

We next attempted to improve the inhibitory potency of the α-keto esters by changing the nature of the N-protecting group on the dipeptide inhibitor and extending the peptide chain. In the RCO-Leu-Abu-COOEt series (compounds 8, 15–22), changing the benzyloxycarbonyl (Z) group to a variety of other acyl moieties led to significantly poorer inhibitors for both calpain I and

Table I. Inhibition of Cysteine Proteases and Platelet Membrane Permeability by Peptidyl α-Keto Esters

no.	compound[a]	K_I (μM) Cal I[b]	Cal II[b]	Cat B[c]	Pap[d]	IC_{50} (μM) plat memb perm[e]
7	Z-Leu-Abu-COOMe		0.50	17		280
8	Z-Leu-Abu-COOEt	4.5	0.40	30	220	300
9	Z-Leu-Abu-COO-*n*-Bu	1.8	0.40	4.0	10	28
10	Z-Leu-Abu-COOBzl	9.5	0.47	4.0	40	100
11	Z-Leu-Phe-COOMe			>100		
12	Z-Leu-Phe-COOEt	1.8	0.40	340	75	200
13	Z-Leu-Phe-COO-*n*-Bu	5.0	1.1	15	15	
14	Z-Leu-Phe-COOBzl	3.4	1.6	45	45	
15	$Ph(CH_2)_2CO$-Leu-Abu-COOEt	40	1.2	23		20
16	$Ph(CH_2)_3CO$-Leu-Abu-COOEt		2.9	23		
17	$Ph(CH_2)_4CO$-Leu-Abu-COOEt		1.9	7.5		
18	$PhCH(CH(CH_3)C_2H_5)CO$-Leu-Abu-COOEt		1.6	24		
19	$PhOCH(C_2H_5)CO$-Leu-Abu-COOEt	20	1.4	14		22
20	2-$NapSO_2$-Leu-Abu-COOEt		2.2	19		
21	Ph_2CHCO-Leu-Abu-COOEt	0.10	0.20			>100
22	$(CH_3)_2CHCH_2OCO$-Leu-Abu-COOEt		17	0.40		>300
23	Z-Leu-Nva-COOEt	1.4	1.2	25	150	100
24	Z-Leu-Nle-COOEt	7.0	0.18	20	190	150
25	Z-Leu-Met-COOEt	1.0	1.5	55	140	
26	Z-Leu-4-Cl-Phe-COOEt	<4.0	0.40	50	150	
27	Z-Leu-Leu-Abu-COOEt	1.8	2.6	22		40
28	Tos-Leu-Leu-Abu-COOEt	33	0.26	69		30
29	2-$NapSO_2$-Leu-Leu-Abu-COOEt	16	1.4	25		100
30	2-NapCO-Leu-Leu-Abu-COOEt	1.3	0.09			78
31	Z-Leu-Leu-Phe-COOEt	1.4	1.9	42	15	

[a] All of the peptide keto ester inhibitors are diasteromeric at the α-carbon of the P_1 residue. [b] 25 mM Tris pH 8.0, 10 mM $CaCl_2$, 5% DMSO. [c] 20 mM sodium acetate pH 5.2, 0.5 mM dithiothreitol, 2% DMSO. [d] 100 mM K_3PO_4, 1 mM EDTA, 2.5 mM cysteine pH 6.0, 2% DMSO. [e] 0.137 M NaCl, 3 mM KCl, 0.4 mM NaH_2PO_4, 12 mM $NaHCO_3$, 1 mM $MgCl_2$, 14.7 mM HEPES, 20 mM glucose, pH 7.35, 2% DMSO.

calpain II, with one exception. The dipeptide **21** with RCO = Ph_2CHCO is the best inhibitor for both calpain I and calpain II with K_I values respectively of 0.1 and 0.2 μM. Compared to Z-Leu-Abu-COOEt, this was a significant improvement with calpain I (45-fold), but less significant with calpain II (2-fold). Extending the dipeptide to a tripeptide α-keto ester resulted in a 3-fold improvement with calpain I in the case of the 2-NapCO (**30**) and Z (**31**) derivatives of Leu-Leu-Abu-COOEt. Interestingly, both the Tos (**28**) and 2-$NapSO_2$ (**29**) derivatives were substantially poorer. The tripeptide **30** is the best α-keto ester inhibitor of calpain II with a K_I value of 0.09 μM, which is 4-fold better than the dipeptide **8**. Overall, we did not consider the increases in potency of enough significance to warrant further pursuit of tripeptide inhibitors for the calpains.

We next evaluated the effect on inhibitory potency of changes in the ester functional group. Changing the ethoxy group to butoxy (**9**) resulted in a slight improvement with calpain I, but no significant change with calpain II. However, **9** was the best inhibitor in the Z-Leu-Abu-COOR series for all of four cysteine proteases with K_I values = 1.8, 0.40, 4, and 10 μM, respectively. It was 5-fold more effective than **10** (R = Bzl) for calpain I, 7-fold better than **8** (R = Et) for cathepsin B, and 22-fold more potent than **8** for papain. No significant improvement of inhibitory potency for calpain II was shown by changing the ester group in Z-Leu-Abu-COOR (K_I = 0.4–0.5 μM). Compound **12** (R = Et) was the best inhibitor in the Z-Leu-Phe-COOR series (compounds **11–14**) for both calpain I (K_I = 1.8 μM) and calpain II (K_I = 0.40 μM). In addition, **12** was the poorest inhibitor for cathepsin B and was 23-fold less effective than **13** (R = *n*-Bu, K_I = 15 μM).

The α-keto esters have considerable specificity for calpain I and II relative to cathepsin B and papain. In general, the inhibitors were 3–190-fold and 36–850-fold better inhibitors respectively of calpain I and calpain II compared to cathepsin B. In general all keto esters were >10-fold better inhibitors of the calpains when compared to papain. However, we did discover some potent cathepsin B inhibitors such as $(CH_3)_2CHCH_2OCO$-Leu-Abu-COOEt (**22**, K_I = 0.4 μM). Compound **22** was also one of the poorest inhibitors for calpain II. While little improvement in the inhibitory potency for calpain I and calpain II was obtained by extending the chain from a dipeptidyl α-keto ester to a tripeptidyl α-keto ester, this modification resulted in a 8-fold improvement in potency for cathepsin B and a 5-fold improvement for papain when comparing Z-Leu-Phe-COOEt to Z-Leu-Leu-Phe-COOEt.

Peptidyl α-Keto Amides. When the α-keto esters were initially tested *in vivo*, we found the compounds were being rapidly degraded probably due to cleavage by plasma esterases. Therefore, we decided to focus further efforts on the more stable α-keto amides, and the inhibitory activities of these derivatives are summarized in Table II. Addition of alkyl or arylalkyl substituents to the nitrogen of the dipeptidyl α-keto amide often resulted in improved inhibitory potency for calpain I and calpain II which indicates the presence of hydrophobic residues in the S′ subsites of both calpains. In the Z-Leu-Abu-CONHR series of inhibitors (**32–49**), most of the best inhibitors for calpain I (K_I = 0.1–0.2 μM; **35**, R = *n*-Bu; **39**, R = $(CH_2)_6CH_3$; **41**, R = $(CH_2)_8CH_3$; **44**, R = Bzl; **45**, R = $(CH_2)_2Ph$; **49**, R = CH_2CHPh_2) and calpain II (K_I = 0.019–0.022 μM; **40**, R = $(CH_2)_7CH_3$; **45**, R = $(CH_2)_2Ph$) have rather hydrophobic substituents. Compound **46** (R = $(CH_2)_3$-Ph) was the best inhibitor for cathepsin B (K_I = 0.2 μM) which we discovered and was substantially better than most of the other *N*-alkyl or arylalkyl derivatives we examined. In the series Z-Leu-Phe-CONHR of inhibitors (**50–56**), compound **56** (R = $(CH_2)_2Ph$) was the best inhibitor for both calpain I (K_I = 0.052 μM) and calpain II (K_I = 0.024 μM). When the hydrophobic group was longer than eight carbon atoms, both the solubility of the peptide α-keto amide in aqueous buffer and the inhibitory potency decreased (**41–43**).

Table II. Inhibition of Cysteine Proteases and Platelet Membrane Permeability by Peptide α-Keto Amides and α-Keto Acids

no.	compound[a]	K_I (μM) Cal I[b]	Cal II[b]	Cat B[c]	Pap[d]	IC_{50} (μM) plat memb perm[e]
32	Z-Leu-Abu-$CONH_2$	0.28	0.019	3.4	190	89
33	Z-Leu-Abu-CONHEt	0.25	0.21	2.4	93	100
34	Z-Leu-Abu-CONH-*n*-Pr		0.25	8.0		70
35	Z-Leu-Abu-COHN-*n*-Bu	0.20	0.05	13	93	35
36	Z-Leu-Abu-CONH-*i*-Bu	4.0	0.14	4.0		28
37	Z-Leu-Abu-$CONHCH(CH_3)C_2H_5$	2.0		0.50		
38	Z-Leu-Abu-$CONHCH_2C_6H_{11}$	0.68	0.044			66
39	Z-Leu-Abu-$CONH(CH_2)_6CH_3$	0.096	0.067	14		
40	Z-Leu-Abu-$CONH(CH_2)_7CH_3$	0.32	0.019	2.6		200
41	Z-Leu-Abu-$CONH(CH_2)_8CH_3$	0.12		150		
42	Z-Leu-Abu-$CONH(CH_2)_9CH_3$		0.60	5.0		
43	Z-Leu-Abu-$CONH(CH_2)_{17}CH_3$	insol				
44	Z-Leu-Abu-CONHBzl	0.20	0.35	2.0		30
45	Z-Leu-Abu-$CONH(CH_2)_2Ph$	0.20	0.022	1.3	38	50
46	Z-Leu-Abu-$CONH(CH_2)_3Ph$	0.80	0.043	0.20		100
47	Z-Leu-Abu-$CONHCH_2CH(CH_3)Ph$	0.32	0.059			27
48	Z-Leu-Abu-$CONH(CH_2)_4Ph$	0.32		1.2		
49	Z-Leu-Abu-$CONHCH_2CHPh_2$	0.16	0.063	3.0		
50	Z-Leu-Phe-$CONH_2$	1.0	0.76	32		
51	Z-Leu-Phe-CONHEt	0.20	0.039	6.0	45	22
52	Z-Leu-Phe-CONH-*n*-Pr	15.0	0.05	3.0		31
53	Z-Leu-Phe-CONH-*n*-Bu	0.50	0.028	3.0		38
54	Z-Leu-Phe-CONH-*i*-Bu	0.08	0.065	4.0		22
55	Z-Leu-Phe-CONHBzl		0.046	4.6		>300
56	Z-Leu-Phe-$CONH(CH_2)_2Ph$	0.052	0.024	9.3	100	100
57	Z-Leu-Nva-$CONH_2$	0.069	0.078			
58	Z-Leu-Nva-CONHEt	0.21	0.088	3.3		40
59	Z-Leu-Abu-$CONEt_2$	insol.		270		
60	Z-Leu-Abu-$CON(n\text{-}Bu)_2$	insol.				
61	Z-Leu-Abu-$CON(Et)CH_2Ph$	insol.		>400		
62	Z-Leu-Abu-$CON(CH_2Ph)_2$	>5		31		
63	Z-Leu-Phe-$CONEt_2$	76				
64	Z-Leu-Phe-COOH	0.0085	0.0057	4.5	7.0	100
65	Z-Leu-Abu-COOH	0.075	0.022	1.5	13.5	100

[a] All of the peptide keto amide inhibitors are diasteromeric at the α-carbon of the P_1 residue. [b] 25 mM Tris pH 8.0, 10 mM $CaCl_2$, 5% DMSO. [c] 20 mM sodium acetate pH 5.2, 0.5 mM dithiothreitol, 2% DMSO. [d] 100 mM K_3PO_4, 1 mM EDTA, 2.5 mM cysteine pH 6.0, 2% DMSO.[e] 0.137 M NaCl, 3 mM KCl, 0.4 mM NaH_2PO_4, 12 mM $NaHCO_3$, 1 mM $MgCl_2$, 14.7 mM HEPES, 20 mM glucose, pH 7.35, 2% DMSO.

In the dipeptide α-keto amides Z-Leu-AA-CONHEt, changing the AA residue to Abu, Nva, or Phe had little effect on the inhibitory potency toward either calpain I (K_I = 0.20–0.25 μM), calpain II (K_I = 0.039–0.21 μM), or cathepsin B (K_I = 2.4–6.0 μM). N-Monosubstituted peptidyl α-keto amides were much more effective inhibitors for calpain I, calpain II, and cathepsin B than the corresponding peptidyl α-keto esters; for example, Z-Leu-Abu-CONHBzl (**44**, K_I = 0.2 μM) was 48-fold better than Z-Leu-Abu-COOBzl (**10**) for calpain I, Z-Leu-Phe-CONHBzl (**55**, K_I = 0.046 μM) was 35-fold better than Z-Leu-Phe-COOBzl (14) for calpain II, Z-Leu-Phe-CONHEt (**51**, K_I = 6 μM) was 57-fold better than Z-Leu-Phe-COOEt (**12**) for cathepsin B. This observation points to the presence of a hydrogen bond donor in the S_1' subsite of cysteine proteases which may be interacting with the N–H on the keto amide functional group. In order to evaluate this hypothesis, we synthesized some representative N,N-disubstituted α-keto amides and found them to be much less potent inhibitors than the N-monosubstituted α-keto amides. For example, compound Z-Leu-Abu-CON(Et)-CH_2Ph (**61**) was 167-fold poorer inhibitor of cathepsin B than compound Z-Leu-Abu-CONHEt (**33**) and 200-fold poorer than Z-Leu-Abu-CONHBzl (**44**). With calpain I, compound Z-Leu-Phe-$CONEt_2$ (**63**) was 380-fold less effective than Z-Leu-Phe-CONHEt (**51**).

Peptidyl α-Keto Acids. Peptide α-keto acids were better inhibitors than the corresponding α-keto esters and α-keto amides for calpain I and calpain II. Indeed, Z-Leu-Phe-COOH (**64**) was the best inhibitor for calpain I and calpain II that we discovered in this study, and Z-Leu-Abu-COOH (**65**) was also among the best for both enzymes. The α-keto acid **64** (K_I = 0.0085 μM was 120–210-fold better than the simple amide Z-Leu-Phe-$CONH_2$ (**50**) and the simple ester Z-Leu-Phe-COOEt (**12**) with calpain I. With calpain II (K_I = 0.0057 μM), it was 70–130-fold more effective than the simple amide and ester. Z-Leu-Abu-COOH (**65**) was also a significantly better inhibitor for calpain I (K_I = 0.075 μM) than the corresponding simple amide (**32**) and ester (**8**), while with calpain II, it was equally potent as the simple amide, but better than the simple ester. The keto acids were substantially poorer inhibitors of cathepsin B than calpain I and calpain II, but were still better inhibitors of cathepsin B than the simple ester and amide. For example, with cathepsin B, Z-Leu-Phe-COOH (**64**, K_I = 4.5 μM) was respectively 76-fold and 7-fold better than the corresponding simple ester Z-Leu-Phe-COOEt (**12**) and amide Z-Leu-Phe-$CONH_2$ (**50**). Z-Leu-Abu-COOH (**65**, K_I = 1.5 μM) was 20-fold better than Z-Leu-Abu-COOEt (**8**) and only 2-fold better than Z-Leu-Abu-$CONH_2$ (**32**). Z-Leu-Abu-COOH was a poor inhibitor of bovine chymotrypsin and porcine pancreatic elastase with K_I > 150 μM, and Z-Leu-Phe-COOH was also a poor inhibitor with K_I values of 76 and >150 μM respectively.

Inhibitory Mechanism. In the course of the kinetic studies with calpain, we observed that several of the inhibitors were exhibiting slow binding behavior which is frequently observed with tightly bound low molecular weight protease inhibitors. A typical example with calpain I is shown in Figure 1. With the inhibitor Z-Leu-Phe-

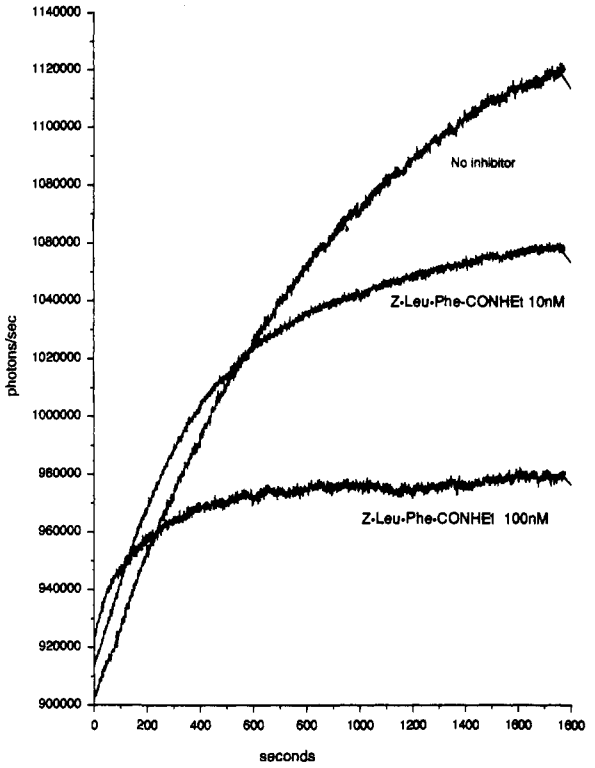
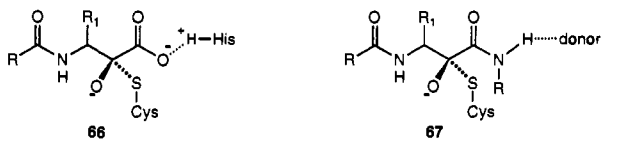


Figure 1. Fluorometer tracing of the hydrolysis of Suc-Leu-Tyr-AMC (1 mM) by calpain I (5 nM) in the presence and absence of Z-Leu-Phe-CONHEt (50 and 100 nM). The plot shows photon counts/s which in this range is linearly related to AMC concentration vs time (s). The enzyme alone will slowly lose activity due to autolysis and repeated measurement of this rate yields k_{obs} values of (1.1–1.8) × 10^{-3} s^{-1}.

CONHEt, slow binding behavior is very evident at 100 nM concentration (k_{obs} = 5.1 × 10^{-3} s^{-1}), while at a lower concentration (10 nM) it is less evident (k_{obs} = 2.4 × 10^{-3} s^{-1}). Slow binding behavior was also observed with Z-Leu-Abu-CONHEt and Z-Leu-Nva-CONHEt where k_{obs} values of 2.5 × 10^{-3} and 2.6 × 10^{-3} s^{-1} were measured, respectively, at 50 nM inhibitor concentration. Slow binding behavior has previously been observed with other tight-binding transition-state inhibitors of proteases. Examples include the binding of peptide keto acids and keto esters to papain and cathepsin B,[22] peptide boronic acids to serine proteases,[34] and peptide phosphinic acid analogues to the aspartic protease pepsin.[35] In the case of papain and cathepsin B, slow binding behavior was observed with Z-Phe-Gly-$COOC_6H_{13}$.

A number of peptide derivatives containing electronegative carbonyl groups have been found to be transition-state inhibitors for cysteine proteases. These structures include peptide aldehydes, peptide α-keto esters, α-keto amides, α-keto acids, and peptide trifluoromethyl ketones. In each case, the active site cysteine of the enzyme is thought to add to the inhibitor carbonyl group to form a hemithioacetal or hemithioketal structure which resembles the tetrahedral adduct formed during substrate hydrolysis. It is also likely that the active site histidine is hydrogen bonded to the carboalkoxy, carboxamido, or carboxylic acid function group, respectively, in α-keto esters, α-keto amides, and α-keto acids. Such an interaction has been observed in the APPA complex with the serine protease trypsin.

Two unusual aspects of our data are the exceptional inhibitory potency of the α-keto acids toward calpains



66 67

Figure 2. Proposed binding mode of a peptide α-keto acid (66) and a monosubstituted α-keto amide (67) to the active site of calpain.

and the observation that monosubstituted amides are much more potent than the corresponding disubstituted amides. In case of the keto acids, we suggest that the active site histidine of the calpains is interacting electrostatically with the terminal carboxyl group of the inhibitor (Figure 2, 66). This structure would be analogous to the structure observed in the APPA-trypsin complex. Clearly, in the case of keto esters and keto amides, this electrostatic interaction could be replaced by a hydrogen bond and may not be sufficient to explain the increased potency toward calpain of α-keto acids. In the case of calpain, it is possible that the calcium atom plays a mechanistic role in substrate hydrolysis and may be indirectly stabilizing the oxyanion as has been observed with the phospholipase A2 mechanism.[36] With monosubstituted α-keto amides, it appears that there is an interaction with a hydrogen-bond donor (Figure 2, 67). The active site histidine could function as both a hydrogen bond donor and acceptor, and it is possible that this donor is the histidine. It is also possible that there is another hydrogen-bond donor in this region of the active site and both residues are interacting with the inhibitor. Clearly it will be necessary to obtain a crystal structure of calpain to definitively answer this question.

Platelet Membrane Permeability Assay. We also evaluated the membrane penetrance of a number of the inhibitors in rat platelets. Treatment of platelets with calcium ionophore results in the elevation of intracellular calcium, activation of calpain, and calpain-mediated cleavage of the cytoskeletal proteins including spectrin. Using spectrin cleavage as an indicator of calpain inhibition, the membrane penetrance of the inhibitors can be inferred. In general the most effective inhibitors in this assay were fairly hydrophobic derivatives. Both α-keto acids were quite poor, and the best derivatives in this assay were Z-Leu-Phe-CONHEt, Z-Leu-Phe-CONH-*i*-Bu, Z-Leu-Abu-COO-*n*-Bu, $Ph(CH_2)_2CO$-Leu-Abu-COOEt, $PhOCH(C_2H_5)CO$-Leu-Abu-COOEt, and Z-Leu-Abu-$CONHCH_2CH(CH_3)Ph$.

Conclusion

We have developed a series dipeptide α-keto esters, α-keto amides, and α-keto acids for the cysteine proteases calpain I and calpain II. In general, α-keto acids were more inhibitory than α-keto amides, which in turn were more effective than corresponding α-keto esters. The more hydrophobic derivatives were also effective in a membrane permeability assay which measures the breakdown of spectrin in platelets. Z-Leu-Phe-COOH (64) was the best inhibitor of calpain I (K_I = 0.0085 μM) and calpain II (K_I = 0.0057 μM), Z-Leu-Abu-$CONH(CH_2)_3Ph$ (46) was the best inhibitor of cathepsin B (K_I = 0.2 μM), and Z-Leu-Abu-COO-*n*-Bu (9) was the best inhibitor of papain (K_I = 10 μM) discovered in this study. A number of the inhibitors were equally potent with the calpains (e.g. 64) but were 1 order of magnitude less effective with cathepsin B. Numerous derivatives were potent calpain II inhibitors

Table III. Physical Properties of α-Keto Esters, α-Keto Amides, and α-Keto Acids

compd no.	mp, °C	% yield[a] (solvent)[c]	TLC R_f (solvent)[d]	formula	MS[b] *m/e* ($M^+ + 1$)	anal.
7	(oil)	53 (B)	0.62 (B)	$C_{20}H_{28}N_2O_6$	393.1991	nd[e]
8	(oil)	73 (B)	0.56 (B)	$C_{21}H_{30}N_2O_6$	407.2134	nd
9	(oil)	43 (B)	0.53 (B)	$C_{23}H_{34}N_2O_5$	435.2481	nd
10	(oil)	42 (B)	0.51 (B)	$C_{26}H_{32}N_2O_6$	469.2153	nd
11	(oil)	45 (B)	0.48 (B)	$C_{25}H_{30}N_2O_6$	455.2171	nd
12	(oil)	64 (B)	0.44 (B)	$C_{26}H_{32}N_2O_6$	469.2053	nd
13	(oil)	53 (B)	0.54 (B)	$C_{28}H_{36}N_2O_6$	497.2591	nd
14	117–110	42 (B)	0.47 (B)	$C_{31}H_{34}N_2O_6$	531.2710	C,H,N
15	(ss)[f]	64 (E)	0.45 (E)	$C_{22}H_{32}N_2O_5$	405.2431	nd
16	(ss)	49 (E)	0.44 (E)	$C_{23}H_{34}N_2O_5$	419.2544	nd
17	(ss)	67 (F)	0.44 (E)	$C_{24}H_{36}N_2O_5$	433.2656	nd
18	(ss)	48 (G)	0.45 (E)	$C_{25}H_{36}N_2O_5$	447.2839	nd
19	(ss)	37 (H)	0.42 (E)	$C_{23}H_{34}N_2O_6$	435	nd
20	(ss)	38 (B)	0.62 (B)	$C_{23}H_{30}N_2O_6S$	463.1944	nd
21	(oil)	30 (J)	0.50 (E)	$C_{27}H_{34}N_2O_5$	467.2511	nd
22	(oil)	61 (J)	0.52 (E)	$C_{16}H_{32}N_2O_6$	373.2347	nd
23	(oil)	74 (B)	0.51 (B)	$C_{21}H_{32}N_2O_6$	421.2264	nd
24	(oil)	53 (B)	0.57 (B)	$C_{23}H_{34}N_2O_6$	435.2230	nd
25	(oil)	46 (B)	0.52 (B)	$C_{22}H_{32}N_2O_6S$	453.2013	nd
26	(oil)	69 (K)	0.71 (K)	$C_{26}H_{31}ClN_2O_6$	503.1931	nd
27	(oil)	79 (B)	0.28 (B)	$C_{27}H_{41}N_3O_6$	520.5004	nd
28	155–160	65 (A)	0.38 (B)	$C_{26}H_{41}N_3O_7S$	540.2712	nd
29	(ss)	61 (D)	0.67 (D)	$C_{29}H_{41}N_3O_7S$	576.2696	nd
30	(ss)	69 (B)	0.42 (C)	$C_{30}H_{41}N_3O_6$	540.3107	nd
31	(oil)	33 (B)	0.56 (B)	$C_{32}H_{43}N_3O_7$	582.3334	nd
32	126–127	31 (C)	0.60 (C)	$C_{19}H_{27}N_3O_5$	378	C,H,N
33	130–132	64 (B)	0.36 (B)	$C_{21}H_{31}N_3O_5$	406	C,H,N
34	134–135	47 (B)	0.28 (B)	$C_{22}H_{33}N_3O_5$	420	C,H,N
35	135–136	42 (B)	0.54 (B)	$C_{23}H_{35}N_3O_5$	434	C,H,N
36	133–135	65 (C)	0.25 (B)	$C_{23}H_{35}N_3O_5$	434	C,H,N
37	91–93	30 (B)	0.30 (B)	$C_{23}H_{35}N_3O_5$	434	C,H,N
38	147–148	43 (C)	0.52 (C)	$C_{26}H_{39}N_3O_5$	474	C,H,N
39	132–133	59 (C)	0.56 (C)	$C_{26}H_{41}N_3O_5$	476	C,H,N
40	134–135	67 (C)	0.55 (C)	$C_{27}H_{43}N_3O_5$	490	C,H,N
41	133–134	49 (C)	0.54 (C)	$C_{28}H_{45}N_3O_5$	504	C,H,N
42	128–130	63 (C)	0.54 (C)	$C_{29}H_{47}N_3O_5$	518	C,H,N
43	134–136	12 (C)	0.54 (C)	$C_{37}H_{63}N_3O_5$	630	C,H,N
44	140–141	29 (C)	0.56 (C)	$C_{26}H_{33}N_3O_5$	468	C,H,N
45	156–157	51 (C)	0.44 (C)	$C_{27}H_{35}N_3O_5$	482	C,H,N
46	142–144	59 (C)	0.45 (C)	$C_{28}H_{37}N_3O_5$	496	C,H,N
47	146–147	49 (C)	0.61 (C)	$C_{28}H_{37}N_3O_5$	496	C,H,N
48	142–144	62 (C)	0.47 (C)	$C_{29}H_{39}N_3O_5$	510	C,H,N
49	145–147	48 (B)	0.51 (B)	$C_{33}H_{39}N_3O_5$	558	C,H,N
50	149–151	55 (C)	0.48 (C)	$C_{24}H_{29}N_3O_5$	440	C,H,N
51	145–147	75 (C)	0.60 (C)	$C_{26}H_{33}N_3O_5$	468	C,H,N
52	152–153	92 (B)	0.50 (B)	$C_{27}H_{36}N_3O_5$	482	C,H,N
53	152–153	67 (B)	0.50 (B)	$C_{28}H_{37}N_3O_5$	496	C,H,N
54	152–153	53 (B)	0.54 (B)	$C_{28}H_{37}N_3O_5$	496	C,H,N
55	160–162	40 (C)	0.45 (C)	$C_{31}H_{35}N_3O_5$	530	C,H,N
56	151–153	50 (C)	0.50 (C)	$C_{32}H_{37}N_3O_5$	544	C,H,N
57	118–121	33 (C)	0.31 (C)	$C_{20}H_{29}N_3O_5$	392	C,H,N
58	143–145	58 (B)	0.36 (B)	$C_{22}H_{33}N_3O_5$	420	C,H,N
59	(oil)	30 (C)	0.63 (C)	$C_{23}H_{35}N_3O_5$	434.4210	nd
60	(oil)	43 (C)	0.70 (C)	$C_{27}H_{43}N_3O_5$	490.3681	nd
61	(ss)	63 (C)	0.73 (C)	$C_{28}H_{37}N_3O_5$	496.2788	nd
62	(ss)	50 (C)	0.76 (C)	$C_{33}H_{39}N_3O_5$	558.2770	nd
63	(ss)	52 (E)	0.66 (E)	$C_{28}H_{37}N_3O_5$	496.3124	nd
64	(ss)	78 (I)	0.28 (I)	$C_{24}H_{28}N_2O_6$	441.2001	nd
65	(ss)	83 (I)	0.21 (I)	$C_{19}H_{28}N_2O_6$	379.1806	nd

[a] Yields are for the conversion of the peptide enol ester to keto ester for keto esters; from keto ester to keto amide for keto amides; from keto ester to keto acid for keto acids. [b] Compounds which are oils or semisolids have high-resolution mass spectral data (FAB), while the solids have C, H, and N analyses. [c] Solvent in the purification of the product by column chromatography. [d] Solvent used in the TLC. [e] nd is not determined. [f] ss is semisolid.

(e.g. **32**) while being at least 10-fold less potent with calpain I, and orders of magnitude less effective with cathepsin B. However, we only discovered a few inhibitors (**21** and **44**) which were more effective with calpain I than calpain II. Clearly the inhibitors reported in this paper should be invaluable for elucidating the role of calpain in various disease states that involve neurodegeneration.

Experimental Section

Chemistry. Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. The purity of each compound was checked by TLC, melting point, and ^1H NMR and mass spectroscopy. TLC was performed on Baker Si250F silica gel plates. Melting points were obtained on a Büchi capillary apparatus and are uncorrected. ^1H NMR spectra were determined on a Varian Gemini 300 instrument Chemical shifts are expressed in ppm relative to internal tetramethylsilane. Mass spectra were obtained on a Varian MAT 112S spectrometer. Column chromatography was performed on silica gel (32–63 μm) using the following solvent systems: A, $CHCl_3/CH_3OH$, 100:1; B, $CHCl_3/CH_3OH$, 50:1; C, $CHCl_3/CH_3OH$, 20:1; D, AcOEt/AcOH, 200:1; E, AcOEt/petroleum ether, 1:1; F, AcOEt/petroleum ether, 9:11; G, AcOEt/$CHCl_3$, 2:3; H,

Table IV. Proton NMR Data ($CDCl_3$) for Selected Compounds

$C_6H_5CH^a_2OCO\text{-}NH^b\text{-}CH^c(R_1)\text{-}CO\text{-}NH^d\text{-}CH^e(R_2)\text{-}CO\text{-}R_3$
$R_1 = CH_2CH(CH_3)_2$; $R_2 = C_2H_5$ or $CH_2C_6H_4$; R_3 = OH, $COOR_4$, $CONH^fR_5$, or COOH

compd no.	H^a	H^b	H^c	H^d	H^e	H^f	other major signals
6	5.09, s	7.17, br d	4.56, m	5.80, br d	4.53, m		8.75–8.45 (b, 1H, OH)
8	5.10, s	6.63, br s	4.23, m	5.18, m	4.23, m		4.36 (q, 2H), 1.37 (t, 3H)
12	5.09, s	6.55, br s	5.03, m	5.41, br s	4.14, m		3.23 and 3.06 (dm, 2H, CH_2Ph) 4.30 (q, 2H), 1.35 (t, 3H)
32	5.09, s	6.58, br s	5.20, m	5.74, m	4.33, m	7.10, br d	
33	5.12, s	6.67 br s	5.16, m	5.26, m	4.23, m	6.87, br s	1.20 (t, 3H), 3.35 (q, 2H)
38	5.10, s	7.00, br d	5.23, m	5.52, m	4.29, m	7.09, br d	1.20 (m, 4H), 1.40–1.80 (m, 11H) 3.13 (t, 2H)
40	5.12, s	6.81, br s	5.25, m	5.32, d	4.26, m	6.94, br s	0.88 (t, 3H), 1.30–1.40 (m, 10H) 1.53 (m, 2H), 3.28 (q, 2H)
45	5.12, s	6.90, br s	5.23, m	6.64, br s	4.21, m	7.20, br d	2.84 (t, 2H), 3.56 (q, 2H)
51	5.09, s	6.60, br s	5.09, m	5.55, m	4.16, m	6.88, br s	1.21 (t, 3H), 3.37 (q, 2H) 3.32 and 3.11 (dd, 2H, CH_2Ph)
56	5.09, s	6.51, m	5.04, m	5.55, m	4.15, m	6.91, br s	2.88 (t, 2H), 3.64 (q, 2H) 3.30 and 3.10 (dd, 2H, CH_2Ph)
64	5.08, s	6.92, m	5.30, m	6.43, br d	4.20, m		3.25 and 2.98 (dd, 2H, CH_2Ph)
65	5.10, s	6.99, br d	5.45, m	6.36, br d	4.26, m		

AcOEt/$CHCl_3$, 3:7; I, $CHCl_3$/CH_3OH/AcOH, 80:10:5; J, $CHCl_3$; K, AcOEt.

The physical properties of the peptide α-keto ester, α-keto acid, and α-keto amide inhibitors are given in Table III. Proton NMR data for the inhibitors are given in Table IV.

Synthesis of Di- and Tripeptidyl α-Enol Esters (Z-Leu-Abu-α-Enol Ester). To a stirred solution of Z-Leu-Abu-OH (compound 6, 20 g, 57 mmol), DMAP (0.69 g, 5.7 mmol), and pyridine (18 mL, 228 mmol) in anhydrous THF (150 mL) was added ethyloxalyl chloride (13.4 mL, 120 mmol) at a rate sufficient to initiate a reflux. The mixture was refluxed for 4 h and cooled to room temperature, and water (100 mL) was added. The reaction mixture was stirred vigorously for 30 min and extracted with AcOEt (3 × 100 mL). The combined organic layers were washed with H_2O (2 × 100 mL) and saturated NaCl (2 × 100 mL), dried over $MgSO_4$, and concentrated, leaving a dark orange oil. Chromatography on a silica gel column with solvent B afforded 18.76 g (65% yield) of Z-Leu-Abu-enol ester: yellow oil; two spots on TLC (*E* and *Z* isomers), R_f = 0.77 (solvent B); ^1H NMR ($CDCl_3$) δ 0.97 (d, 6H), 1.26 (t, 3H), 1.39 (t, 3H), 1.42 (t, 3H), 1.50–1.85 (m, 3H), 2.86 (q, 2H, C=CCH_2), 4.30 (m, 1H, α-carbon of Leu), 4.36 (q, 2H), 4.45 (q, 2H), 5.15 (s, 2H), 5.20 (m, 1H, NH of Leu), 7.38 (m, 5H), 11.30 (br s, 1H, C=CNH); MS (FAB, calcd for M + 1), $C_{25}H_{35}N_2O_9$, 507) *m/e* 507.

Synthesis of Di- and Tripeptidyl α-Keto Esters (Z-Leu-Abu-COOEt, 8). To a stirred solution of the Z-Leu-Abu-enol ester (20 g, 39.5 mmol) in anhydrous ethanol (100 mL) was added sodium ethoxide (0.272 g, 4 mmol). The solution was stirred for 3 h at room temperature, the ethanol was evaporated, and the residue was treated with ethyl ether (400 mL). The ether layer was washed with H_2O (2 × 100 mL) and saturated NaCl (2 × 100 mL), dried over $MgSO_4$, and concentrated leaving a oil. Chromatography on a silica gel column with solvent B afforded 11.7 g (73% yield) of Z-Leu-Abu-COOEt: a yellow oil; single spot on TLC, R_f = 0.44 (solvent B); MS (FAB, high-resolution, calcd for M + 1, $C_{21}H_{31}N_2O_6$, 407.2182) *m/e* 407.2133.

Synthesis of Compounds 32–37, 50–54, and 57–58 (Z-Leu-Abu-$CONH_2$, 32). The α-carbonyl group of Z-Leu-Abu-COOEt was protected as a 1,3-dithiolane derivative. To a solution of Z-Leu-Abu-COOEt (8, 1 g, 2.46 mmol) in 5 mL of CH_2Cl_2 was added 1,2-ethanedithiol (0.25 g, 2.70 mmol), followed by 0.5 mL of boron trifluoride etherate. The solution was stirred overnight at room temperature. Water (20 mL) and ethyl ether (30 mL) were added. The organic layer was separated, washed with water (2 × 10 mL) and saturated NaCl (2 × 10 mL), dried over $MgSO_4$, and evaporated to afford 1.0 g (84% yield) of a yellow semisolid: ^1H NMR ($CDCl_3$) δ 0.85–0.95 (m, 9H), 1.35 (m, 3H), 1.50–1.70 (m, 4H), 2.00 (m, 1H), 2.60–2.70 (m, 4H, $S(CH_2)_2S$), 4.15–4.30 (m, 4H, OEt and α-carbon of amino acid), 5.10 (s, 2H), 5.22 (br s, 1H, NH of Abu), 6.35 (br s, 1H, NH of Leu), 7.34 (s, 5H); MS (FAB, high-resolution, calcd for M + 1, $C_{23}H_{34}N_2O_5S_2$, 483.1987) *m/e* 483.1972. The protected α-keto ester was used for next reaction without further purification.

The protected Z-Leu-Abu-COOEt (1 g, 2.07 mmol) was dissolved in ethanol (5 mL) and cooled to 0–5 °C in an ice bath, and NH_3 (0.35 g, 20.7 mmol) was bubbled into the solution. With ammonia and gaseous amines, we used 10 equiv of the amine. With solid and liquid amines, we used 3 equiv of the amine. The reaction mixture was allowed to warm to room temperature slowly, and stirred overnight. The mixture was filtered to remove the white precipitate, leaving a yellow semisolid after evaporation of the solvent. Chromatography on a silica gel column using solvent C, followed by precipitation using AcOEt/hexane, afforded 0.24 g (31% yield) of Z-Leu-Abu-$CONH_2$: pale yellow solid; single spot on TLC, R_f = 0.60 (C); mp 126–127 °C; ^1H NMR (DMSO) consistent with the proposed structure; MS (FAB, calcd for M + 1, $C_{19}H_{28}N_3O_5$, 378) *m/e* 378.

Synthesis of Compounds 38–49, 55–56 (Z-Leu-Abu-$CONHCH_2C_6H_{11}$, 38). Compound 38 was synthesized using 3 equiv of cyclohexylmethylamine in place of ammonia in the above procedure. After the mixture was stirred overnight at room temperature, ethyl acetate (60 mL) was added. The mixture was filtered to remove a white precipitate. The solution was then washed with cold 1 M HCl (3 × 25 mL), water (1 × 20 mL), and saturated NaCl (2 × 20 mL) and dried over $MgSO_4$. The solvent was evaporated to get a yellow solid. Chromatography on a silica gel column using solvent C afforded a yellow solid (43% yield): single spot on TLC, R_f = 0.52 (C); mp 147–148 °C; ^1H NMR ($CDCl_3$) consistent with the proposed structure; MS (FAB, calcd for M + 1, $C_{28}H_{40}N_3O_5$, 474) *m/e* 474.

Synthesis of N,N-Disubstituted Keto Amides, Compounds 59–63 (Z-Leu-Abu-$CONEt_2$, 59). To a stirred solution of Z-Leu-Abu-COOH (65, 1 mmol), diethylamine (1.5 mmol), and HOBt (0.5 mmol) in CH_2Cl_2 (10 mL) was added DCC (1.5 mmol) at 0–5 °C. The mixture was stirred for 1 h at the same temperature, stirred overnight at room temperature, and filtered to remove white precipitate. The yellow solution was washed with 1 M HCl, 10% Na_2CO_3, H_2O, and saturated NaCl, dried over $MgSO_4$, and concentrated. The residue was purified by column chromatography using solvent C to afford 0.13 g (30% yield) of Z-Leu-Abu-$CONEt_2$: white oil; single spot on TLC, R_f = 0.63 (C); ^1H NMR ($CDCl_3$) consistent with the proposed structure; MS (FAB, high-resolution, calcd for M + 1, $C_{23}H_{36}N_3O_5$, 434.4235) *m/e* 434.4210.

Synthesis of Dipeptidyl α-Keto Acids (Z-Leu-Phe-COOH, 64). To a stirred solution of Z-Leu-Phe-COOEt (0.53 g, 1.13 mmol) in CH_3OH (6 mL) was added 1 M NaOH (1.27 mL, 1.27 mmol) at room temperature. After being stirred for 2 h at room temperature, the solution was acidified to pH 3, and AcOEt (50 mL) and saturated NaCl (25 mL) were added. The water phase was extracted with AcOEt (2 × 30 mL). The combined organic layers were washed with water (2 × 50 mL) and saturated NaCl

(2 × 50 mL), dried over $MgSO_4$, and concentrated to yield a colorless semisolid (78% yield): single spot on TLC, R_f = 0.28 (I); ^1H NMR ($CDCl_3$) consistent with the proposed structure; MS (FAB, high-resolution, calcd for M + 1, $C_{24}H_{28}N_2O_6$, 441.2026) *m/e* 441.2000.

Biochemistry. HEPES, heparin, and A23187 were obtained from Calbiochem. The fluorogenic calpain substrate Suc-Leu-Tyr-AMC was obtained from Bachem (Switzerland). Chromogenic substrates including Bz-Phe-Val-Arg-*p*-nitroanilide were purchased from Sigma. Calpain I was purified from human erythrocytes according to the method of Kitahara et al.,[37] omitting the Blue-Sepharose step. Calpain II from rabbit muscle and bovine cathepsin B were purchased from Sigma. Protein (calpain) concentrations were determined using the Bio-Rad protein assay kit (Bradford assay) using bovine serum albumin as a standard. Papain was purchased from Calbiochem. Polyclonal antisera was prepared by immunization of rabbit with rat brain spectrin purified according to Bennett method.[38]

Kinetic Assays. Calpain assays were performed in 25 mM Tris, pH 8.0, 1 mM β-mercaptoethanol, 10 mM $CaCl_2$, 1–3 mM substrate (Suc-Leu-Tyr-AMC[10]) with 70 nM (final concentration) enzyme. Fluorescence of the cleavage product 7-amino-4-methylcoumarin (λ_{ex} = 380 nm, λ_{em} = 460 nm) was followed using a Gilson FL-1A fluorometer, a Perkin-Elmer 203 fluorescence spectrometer, or a SPEX Fluoromax fluorometer.

Inhibitors and substrates were added to reactions as DMSO solutions where the DMSO content of the final mixture did not exceed 5%. Typically, 10 μL of the substrate solution (100 mM stock in DMSO) was added to 1 mL of buffer. The inhibitor solution (10 μL of a DMSO solution of varying concentrations) was then added and the reaction initiated by the addition of 30–50 μL of calpain in the assay buffer but without calcium. After a delay of 30 s, initial velocities (t = 30–60 s) were determined at room temperature at five or more concentrations of inhibitor (not exceeding 2 × K_I(obs)) and at two fixed concentrations of substrate. K_I values were determined by Dixon plots. The average of triplicate assays, plotted as $1/v$ versus I, gave intersecting lines with a correlation coefficient ≥0.95. No other attempt was made to correct for slow binding or autolysis.

Using the above methods, calpain inhibitor I consistently gave a K_I = 0.2 μM for calpain I which is in agreement with Sasaki.[11]

Cathepsin B was assayed in 20 mM sodium acetate pH 5.2, 0.5 mM dithiothreitol using Bz-Phe-Val-Arg-*p*-nitroanilide as substrate. Papain was assayed in 100 mM K_3PO_4, 1 mM EDTA, 2.5 mM cysteine, pH 6.0 using Bz-Arg-*p*-nitroanilide as substrate. Cleavage of *p*-nitroaniline was followed at 405 nm on a Perkin-Elmer Lambda 2 spectrophotometer or a Molecular Devices Thermomax microplate reader.

Platelet Membrane Permeability Assay. Calpain-mediated breakdown of spectrin was measured by quantitative densitometry of the calpain-specific 150/155-kDa spectrin fragment doublet.[39] Platelets were isolated by a modification of the method of Ferrell and Martin.[40] Platelets were isolated from freshly drawn rat blood. Blood collected by cardiac puncture was anticoagulated with 0.05 volumes of 100 mM citrate–EDTA and centrifuged 10 min at 500 *g* at room temperature. The plasma was applied to a 1.5- × 20-cm column of Sepharose 2B (Pharmacia) equilibrated with HEPES-Tyrodes buffer (0.137 M NaCl, 3 mM KCl, 0.4 mM NaH_2PO_4, 12 mM $NaHCO_3$, 1 mM $MgCl_2$, 14.7 mM HEPES, 20 mM glucose, pH 7.35 (Melloni et al.)).[37,41] The column was developed in the same buffer at room temperature, and fractions containing platelets were pooled and adjusted to 10^7 platelets/mL and allowed to rest for a minimum of 10 min at room temperature.

Platelets (100 μL) were incubated with inhibitor (1 μL added from DMSO stocks) for 5 min at room temperature followed by addition of A23187 to a final concentration of 1 μM and calcium to a final concentration of 2 mM. After 5-min exposure the platelets were harvested by centrifugation at 10000 *g* for 10 s, and the pellets were dissolved in SDS-PAGE sample buffer and heated to 90 °C for 3 min.

Platelet samples were subjected to SDS-PAGE in 4–12% gradient gels and transferred to nitrocellulose by electroblotting. Nitrocellulose filters were analyzed for spectrin by standard western blotting. Filters were blocked for 15 min in 0.5% gelatin, 2% bovine serum albumin, 0.9% NaCl, 5 mM sodium phosphate, pH 7.5, and then incubated overnight with antibody to rat brain spectrin in a buffer containing 10 mM Tris, pH 7.5, 0.89% NaCl, and 0.15% Triton X-100 (buffer A). Filters were washed in buffer A without Triton X-100 and then incubated in buffer A containing alkaline phosphatase-conjugated anti-rabbit antibody (BioRad) for 4 h. Filters were washed as above and developed using the BioRad conjugate substrate kit (5-bromo-4-chloro-3-indolyl phosphate, nitroblue tetrazolium, in a diethanolamine buffer, ca. pH 10, 10 mM $MgCl_2$). Quantitative densitometry was used to determine the amount of intact spectrin and 150/155-kDa spectrin breakdown products.

References

(1) Johnson, P. Calpains (Intracellular Calcium-Activated Cysteine Proteinases): Structure-Activity Relationships and Involvement in Normal and Abnormal Cellular Metabolism. *Int. J. Biochem.* **1990**, *22*, 811–822.

(2) Mellgren, R. L.; Murachi, T. *Intracellular Calcium-dependent Proteolysis*; CRC Press: Boca Raton, FL, 1990; pp 1–280.

(3) Melloni, E.; Pontremoli, S. The Calpains. *Trends Neurol. Sci.* **1989**, *12*, 438–444.

(4) Barrett, M. J.; Goll, D. E.; Thompson, V. F. Effect of Substrate on Ca^{+2}-Concentration Required for Activity of the Ca^{2+}-Dependent Proteases, μ- and m-Calpain. *Life Sci.* **1991**, *48*, 1659–1669.

(5) Bartus, R. T. Drugs to Treat Age-related Neurodegenerative Problems. The Final Frontier of Medical Science? *J. Am. Geriatrics Soc.* **1990**, *38*, 680–695.

(6) Zhang, H.; Johnson, P. Effects of Trifluoperazine and Compounds W-7 and W-13 on Chicken Gizzard Calpain II. *Biochem. Soc. Trans.* **1988**, *16*, 1043–1044.

(7) Zhang, H.; Johnson, P. Inhibition of Calpains by Calmidazolium and Calpastatin. *J. Enzyme Inhib.* **1988**, *2*, 163–166.

(8) Johnson, P.; Hammer, J. L. Inhibitory Effects of Spermine and Spermidine on Muscle Calpain II. *Experientia* **1990**, *46*, 276–278.

(9) Rich, D. H. Inhibitors of Cysteine Proteinases. In *Proteinase Inhibitors*; Barrett, A. J., Salvensen, G., Eds.; Elsevier: Amsterdam, 1986; pp 153–178.

(10) Sasaki, T.; Kikuchi, T.; Yumoto, N.; Yoshimura, N.; Murachi, T. Comparative Specificity and Kinetic Studies of Porcine Calpain I and Calpain II with Naturally Occuring Peptides and Synthetic Fluorogenic Substrates. *J. Biol. Chem.* **1984**, *259*, 12489–12494.

(11) Sasaki, T.; Kishi, M.; Saito, M.; Tanaka, T.; Higuchi, N.; Kominami, E.; Katunuma, N.; Murachi, T. Inhibitory Effect of Di- and Tripeptidyl Aldehydes on Calpains and Cathepsins. *J. Enzyme Inhib.* **1990**, *3*, 195–201.

(12) Hori, H.; Yasutake, A.; Minematsu, Y.; Powers, J. C. Inhibition of Human Leukocyte Elastase, Pancreatic Elastase and Cathepsin G by Peptide Ketones. In *Peptides: Structure and Function. Proceedings of the Ninth American Peptide Symposium*; Deber, C. M., Hruby, V. J., Kopple, K. D., Eds.; Pierce Chem. Co.: Rockford, IL, 1985; pp 819–822.

(13) Abbreviations include: Abu, α-aminobutyric acid; AcOEt, ethyl acetate; AMC, 7-amino-4-methylcoumarin; APPA, (4-amidinophenyl)pyruvic acid; *i*-Bu, isobutyl; *n*-Bu, *n*-butyl; Bzl, benzyl; Cal I, calpain I; Cal II, calpain II, Cat B, cathepsin B; DCC, dicyclohexylcarbodiimide; DMAP, 4-(dimethylamino)pyridine; HEPES, *N*-(2-hydroxyethyl)piperazineethanesulfonic acid; HOBt, 1-hydroxybenzotriazole; 2-NapCO, 2-naphthoyl; 2-NapSO_2, 2-naphthylsulfonyl; Nle, norleucine; Nva, norvaline; Pap, papain; *n*-Pr, *n*-propyl; Ph, phenyl; Suc, succinyl; TLC, thin-layer chromatography; Tos, tosyl; Z, benzyloxycarbonyl. The peptidyl keto esters $R_2CONHCH(R_1)COCOOR$ are abbreviated as R_2CO-AA-COOR; the peptidyl keto amides $R_2CONHCH(R_1)COCONHR$ as R_2CO-AA-CONHR; and the peptidyl keto acids $R_2CONHCH(R_1)COCOOH$ as R_1CO-AA-COOH.

(14) Walter, J.; Bode, W. The X-ray Crystal Structure Analysis of the Refined Complex Formed by Bovine Trypsin and *p*-Amidinophenylpyruvate at 1.4 Å Resolution. *Hoppe-Seyler's Z. Physiol. Chem.* **1983**, *364*, 949–959.

(15) Geratz, J. D. *p*-Amidinophenylpyruvic Acid: A New Highly Effective Inhibitor of Enterkinase and Trypsin. *Arch. Biochem. Biophys.* **1967**, *118*, 90–96.

(16) We use the subsite nomenclature of Schechter, I.; Berger, A. On the size of the active site in protease. 1. Papain. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157–162 to describe the individual residues of the inhibitors and the corresponding subsites of the enzyme.

(17) Peet, N. P.; Burkhart, J. P.; Angelastro, M. R.; Giroux, E. L.; Mehdi, S.; Bey, P.; Kolb, M.; Neises, B.; Schirlin, D. Synthesis of Peptidyl Fluoromethyl Ketones and Peptidyl α-Keto Esters as Inhibitors of Porcine Pancreatic Elastase, Huamn Neutrophil Elastase, and Rat and Human Neutrophil Cathepsin G. *J. Med. Chem.* **1990**, *33*, 394–407.

(18) Mehdi, S.; Angelastro, M. R.; Burkhart, J. P.; Koehl, J. R.; Peet, N. P.; Bey, P. The Inhibition of Human Neutrophil Elastase and Cathepsin G by Peptidyl 1,2-Dicarbonyl Derivatives. *Biochem. Biophys. Res. Commun.* **1990**, *166*, 595–600.

(19) Angelastro, M. R.; Mehdi, S.; Burkhart, J. P.; Peet, N. P.; Bey, P. α-Diketones and α-Keto Ester Derivatives of N-Protected Amino Acids and Peptides as Novel Inhibitors of Cysteine and Serine Proteinases. *J. Med. Chem.* **1990**, *33*, 11–13.
(20) Parisi, M. F.; Abeles, R. H. Inhibition of Chymotrypsin by Fluorinated α-Keto Acid Derivatives. *Biochemistry* **1992**, *31*, 9429–9435.
(21) Fusetani, N.; Matsunaga, S.; Matsumoto, H.; Takebayashi, Y. Cyclotheonamides, Potent Thrombin Inhibitors, from a Marine Sponge *Theonella sp.* *J. Am. Chem. Soc.* **1990**, *112*, 7053–7054.
(22) Hu, L. Y.; Abeles, R. H. Inhibition of Cathepsin B and Papain by Peptidyl α-Keto Ester, α-Keto Amides, α-Diketones, and α-Keto Acids. *Arch. Biochem. Biophys.* **1990**, *281*, 271–274.
(23) Huang, Z.; Mcgowan, E. B.; Detwiler, T. C. Ester and Amide Derivatives of E64c as Inhibitors of Platelet Calpains. *J. Med. Chem.* **1992**, *35*, 2048–2054.
(24) Ocain, T. D.; Rich, D. H. α-Keto Amide Inhibitors of Aminopeptidases. *J. Med. Chem.* **1992**, *35*, 451–456.
(25) (a) Brenner, M.; Muller, H. R.; Pfister, R. W. A New Enzymatic Peptide Synthesis. *Helv. Chim. Acta* **1950**, *33*, 568–591. (b) Brenner, M.; Huber, W. Production of α-Aminoacid Esters by Alkoholysis of the Methyl Ester. *Helv. Chim. Acta* **1953**, *36*, 1109–1115.
(26) Rachele, J. R. The Methyl Esterificiation of Amino Acids with 2,2-Dimethoxypropane and Aqueous Hydrogen Chloride. *J. Org. Chem.* **1963**, *28*, 2898–2901.
(27) Bergmann, H.; Zervas, L. A General Method of Peptide Synthesis. *Chem. Ber.* **1932**, *65*, 1192–1201.
(28) Konig, W.; Geiger, R. A New Method for Synthesis of Peptides: Activation of the Carboxyl Group with Dicyclohexylcarbodiimide using 1-Hydroxybenzotriazoles as Additives. *Chem. Ber.* **1970**, *103*, 788–798.
(29) Charles, I.; Latham, D. W. S.; Hartley, D.; Oxford, A. W.; Scopes, D. I. C. Bicyclic Heterocycles with Nitrogen at the Ring Junction. Part 2. Application of the Dakin-West Reaction to the Synthesis of Imidazo-(5,1-f)-1,2,4-triazin-4(3H)-ones. *J. Chem. Soc., Perkin Trans. 1* **1980**, 1139–1146.
(30) Buchanan, G. L. The Dakin-West Reaction. *Chem. Soc. Rev.* **1988**, *17*, 91–109.
(31) Marren, C. B.; Malee, E. J. Synthesis of *n*-Butyl and Benzyl Oxalyl Chloride. *J. Chromatogr.* **1972**, *64*, 219–222.
(32) Sasaki, T.; Kikuchi, T.; Yumoto, N.; Yoshimura, N.; Murachi, T. Comparative Specificity and Kinetic Studies of Porcine Calpain I and Calpain II with Naturally Occuring Peptides and Synthetic Fluorogenic Substrates. *J. Biol. Chem.* **1984**, *259*, 12489–12494.
(33) Takahashi, K. Calpain Substrate Specificity. In *Intracellular Calcium-dependent Proteolysis*; CRC Press: Boca Raton, FL, 1990; pp 55–74.
(34) Kettner, C. A.; Shenvi, A. B. Inhibition of the Serine Proteases Leukocyte Elastase, Pancreatic Elastase, Cathepsin G and Chymotrypsin by Peptide Boronic Acids. *J. Biol. Chem.* **1984**, *259*, 15106–15114.
(35) Bartlett, P. A.; Kezer, W. B. Phosphinic Acid Dipeptide Analogues: Potent, Slow-binding Inhibitors of Aspartic Peptidases. *J. Am. Chem. Soc.* **1984**, *106*, 4282–4283.
(36) Scott, D. L.; White, S. P.; Otwinowski, Z.; Yuan, W.; Gelb, M. H.; Sigle, P. B. Interfacial Catalysis; The Mechanism of Phospholipase A2. *Science* **1990**, *250*, 1541–1546.
(37) Kitahara, A.; Sasaki, T.; Kikuchi, T.; Yomoto, N.; Yoshimura, N.; Hatanaka, M.; Murachi, T. Large-scale Purification of Porcine Calpain I and Calpain II and Comparison of Proteolytic Fragments of Their Subunits. *J. Biochem.* **1984**, *95*, 1759–1766.
(38) Bennett, V.; Baines, A. J.; Davis, J. Brain Analogs of RBC Membrane Proteins: Ankyrin, Protein 4.1 (Synapsin), Spectrin, and Spectrin Subunits. *Methods Enzymol.* **1986**, *34*, 55–69.
(39) Seubert, P.; Ivy, G.; Larson, J.; Lee, J.; Shahi, K.; Baudry, M.; Lynch, G. Lesions of Entorhinal Cortex Produce a Calpain-mediated Degradation of Brain Spectrin in Dentate Gyrus. I. Biochemical Studies. *Brain Res.* **1988**, *459*, 226–232.
(40) Ferrell, J. E., Jr.; Martin, G. S. Thrombin Stimulates the Activities of Multiple Previously Unidentified Protein Kinases in Platelets. *J. Biol. Chem.* **1989**, *264*, 20723–20729.
(41) Meloni, F. J.; Schmaier, A. H. Low Molecular Weight Kininogen Binds to Platelets to Modulate Thrombin-induced Platelet Activation. *J. Biol. Chem.* **1991**, *266*, 6786–6794.